# Oxidative stress responses in hepatocarcinogenesis: unravelling the mechanisms using a toxicogenomics approach

# ADDENDUM

## Valorisation

## Relevance

The focus of this thesis was on gaining a better insight in the carcinogenic impact of oxidant exposure, with particular emphasis on hepatocellular carcinoma (HCC) using a human hepatoma cell line (HepG2). HCC is the predominant primary malignancy of the liver in most countries. It is the fifth most common cancer in men, and seventh among women, and overall the second leading cause of cancer related mortality in the world [1]. Together with the millions of estimated new cases each year, HCC inflicts a high social but also economic burden. Therefore, investing in prevention, early detection and treatment of this disease is of major importance.

Chronic liver disease due to hepatitis B virus (HBV) or hepatitis C virus (HCV) accounts for the majority of HCC cases in developing countries, while in Western Europe and Northern America other risk factors exist such as lifestyle in general (*e.g.*, obesity and diabetes) and exposure to compounds present in the environment, drugs or food [1, 2]. Prevention of HBV is possible by vaccination, while a HCV vaccine is until today not available but in development. The existence and development of such vaccines and the subsequent control of these viral infections, predicts a decrease in the rates of HCC, especially in developing countries [3]. However, as the contributions of HBV and HCV infections diminish, other risk factors present in the environment may become increasingly important as drivers of the future HCC incidence worldwide. Although these different inducers of HCC have different cellular targets and modes of action, their common feature is the formation of oxidative stress [4, 5]. Oxidative stress plays an important role in HCC initiation and progression and is even associated with intrinsic drug resistance [6]. Moreover, oxidative stress does not only induce mutations in DNA but can also induce changes in the DNA methylation status or inflict histone modifications which may further contribute to genomic instability and hepatocarcinogenesis [7-11].

Already decades ago, it is hypothesized that antioxidants can be used to scavenge these reactive oxygen species (ROS) and thus reduce damage. However, in large clinical trials [12-15], the effects of antioxidants could not be proven, on the contrary, they can even be harmful since ROS are also necessary to maintain normal cellular physiology [16]. For that reason, it is necessary to increase our understanding on the underlying molecular responses of the transcriptome and epigenome towards oxidative stress-induced damage and how this can be recognized, by for example specific gene signatures, to improve the risk assessment of new and existing chemicals, such as potential carcinogens and drugs.

## Target groups

The knowledge obtained in this thesis can be used for further translational and applied research purposes, for instance for developing *in vitro* tests in the

pharmaceutical, food and chemical industry. These may increase the correctness of prediction, since an incorrect prediction of human safety increases the risk of admitting dangerous chemicals (in drugs, pesticides, food, *etc*…) to the market. On the other hand, incorrect prediction, due to differences in toxic responses between rodents and human, might also lead to the rejection of truly innocent compounds from the market, from which consumers can benefit, for instance as a drug or food additive.

In the pharmaceutical industry, before new drugs proceed into clinical trials, preclinical testing is performed, which mostly consists out of long-term animal experiments. If a drug does not induce adverse effects in these *in vivo* animal tests, the drug will be tested further in clinical trials using patients and healthy individuals. Roughly 20% of all newly developed drugs which precede this far in the pipeline of drug development are proven successful and become publically available [17]. This complete process of development and approval of drugs may cost up to US $800 million before they are released onto the market, what makes it a time-and money-consuming business [18]. Moreover, despite the existing toxicity testing procedures of new drugs, some drugs available on the market are identified to be hepatotoxic and can even induce HCC (*e.g.*, Bromfenac and Troglitazone). This can be induced by active metabolites or the formation of oxidative stress during metabolization of specific drugs, which makes hepatotoxicity one of the major reasons for drug withdrawal [2]. Also the lack of knowledge in epigenetic processes which can be induced by different oxidative stress-inducing compounds can contribute to such adverse outcomes in later phases of drug development. Additionally, many drugs are pharmacological hepatotoxic (=dose dependent, *e.g.*, Acetaminophen) which are unlimitedly available without a doctor's prescription [19]. This can have tremendous effects on population's health but also have a disastrous effect for a company's credibility and economic status. This indicates that toxicity testing in the pharmaceutical industry needs to be improved to reduce the number of falsely predicted compounds and subsequent economic and social costs.

The same applies to the food and chemical industry. Both have to follow strict rules and regulations in the development and use of their products to ensure human and environmental safety. Risks caused by chemicals (*e.g.*, pesticides, metals, …) and food additives (*e.g.*, food dyes, flavor enhancers, nanoparticles, …) have to be assessed, managed, and communicated to the customer by correctly labeling their products [20]. This process is strictly regulated by initiatives developed by the European Union, called REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) and OECD (Organization for Economic Co-operation and Development) [21, 22]. These authorities apply different programs to increase the safety of different chemicals. Because the current *in vivo* toxicity tests for human safety using rodents come along with a financial burden and ethical dilemmas, are not high-throughput and are questioned for their reliability towards the human situation [23], REACH and OECD also promote the development of alternatives for these animal tests. Mechanistic information discussed in this thesis can contribute to the development of new toxicity screening

methods and, since our results are obtained by *in vitro* cell models, it can contribute to the 3 R's (Reduction, Refinement and Replacement) in animal testing.

In general, the knowledge obtained in this thesis can contribute to the improvement of the risk assessment of such drugs, chemicals and food additives, but can also contribute to a more targeted approach in drug development in oxidative stress-driven cancers. By early prediction of toxicity induced via oxidative stress and better molecular understanding of this mechanism by compounds present in drugs, food and the environment, several future cases of HCC can be avoided which will lead to less human suffering and a lower economic burden.

## Activities/products

Knowledge obtained in this thesis is mainly fundamental and describes mechanisms of oxidative stress in the development of HCC that can be used in more translational and applied research. Our results can create new opportunities for implementation of this knowledge in toxicological screening tests for investigating potential harmful or beneficial effects of new chemicals and drugs. In addition, compounds already present in the environment or pharmaceutical market can be re-evaluated for their oxidative stress-capacities over time which can lead to new information of possible hazardous effects for human or other organisms.

By using extensive time series-analysis for risk evaluation obtained from experiments on cell models in combination with transcriptomics and epigenomics, alternative approaches for animal studies in risk assessment can be developed. This can also service the further enrichment of new analytical tools and statistical approaches to make optimal use of the obtained data. Moreover, new treatment methods can be developed, since unravelling underlying pathways is the first step in the process of new drug development. However, since our knowledge is preliminary, more research is needed before applications of our results can be used to reduce the economic and social burden of HCC.

## Innovation

This research focuses on oxidative stress mechanisms in the formation of liver toxicity and carcinogenesis. Different 'omics techniques such as transcriptomics and (hydroxy-)methylomics were used in combination with detection of phenotypic endpoints such as DNA damage and cell cycle distribution changes in order to unravel these specific oxidative stress-related mechanisms towards cellular damage. Since such oxidative stress-responses will differ in time, we used an innovative temporal analysis to examine time-dependent, sequential changes in gene expression and methylation to provide new insights in the responses towards oxidant challenge.

A combined temporal gene expression profile was identified for menadione and TBH exposure. This cluster consists out of 17 co-expressed genes that are involved in oxidative stress mechanisms and liver carcinogenesis (BIK, AKR1C2, GCLC, GCLM, GSR, LIF, RAP1GAP, SQSTM1, GCNT3, RRAS2, SLC7A11, ASF1A, ASKR1B10, FBXO30, AGPAT9, SRXN1, PTGR1). This specific oxidative stress related gene expression profile can be applied for a better recognition and mechanistic understanding of oxidative stress induced cell damage by chemicals. This gene set was used in Chapter 4 to identify genotoxic-, non-genotoxic- and non-carcinogens that have the ability to induce oxidative stress. We observed that a higher number of upregulated genes is associated with a higher genotoxic capacity of the compound. This demonstrates the relevance of this gene list as well as the use of time series-analysis to predict oxidative stress-inducing capacities of unknown compounds. This temporal gene expression profiles can be further developed by testing the expression of these particular genes in liver tissue and blood samples of HCC patients.

In addition, by combining transcriptomic and epigenomic data from the whole genome following oxidative stress, we were able to identify a counter-regulating property of the methylome towards the primary response of the transcriptome over time. These results further underline that toxicity screening has to be improved by emphasize on time series analysis but also on epigenetic responses towards different compounds.

These findings can eventually open up new doors for prevention and early treatment in HCC and possibly other chronic liver diseases.

## Implementation

Apart from the economic aspect, societal value can be created from knowledge obtained in this thesis. Finding out more about the specific underlying oxidative stress mechanisms in hepatocarcinogenesis can ultimately lead to better prevention and early treatment methods. By using the previously discussed temporal gene expression profile as a diagnostic tool, potential patients can be screened for HCC in an early stage of disease. If the underlying mechanisms are really known, this will also lead to the reduction of side-effects of certain treatments, which is again beneficial for the patient.

As previously discussed, chemical safety is important and increasing the safety of compounds in drugs, environment and nutrition is a task of regulatory authorities such as the OECD. The OECD has recently launched a new program on the generation of Adverse Outcome Pathways (AOP) [24]. A better understanding of oxidative stress-related mechanisms of certain compounds can facilitate the construction of such AOPs. Possibly, an oxidative stress-specific AOP can be implemented in the future. The development of such *in vitro* tests can facilitate the further identification of key events in oxidative stress-related damage

induced by certain compounds. This will reduce the amount of laboratory animals, economic costs, but especially also the social impact of adverse drug effects that can be avoided by a better risk assessments.